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Exome sequencing reveals new insights into the progression of abdominal aortic aneurysm

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Abstract. – BACKGROUND: Abdominal aortic aneurysm (AAA) is a multi-factorial disease and its underlying pathogenesis remains poorly understood.

AIM: We aim to search for the underlying etiology of AAA using whole exome sequencing and gene expression analysis.

MATERIALS AND METHODS: We performed whole exome sequencing for AAA and adjacent normal abdominal aorta tissue from one male AAA patient. Further gene expression analysis using downloaded dataset from the GEO database was also carried out to explore the underlying molecular mechanisms.

RESULTS: A total of 5.97 Gb clean data were generated for the two samples, achieving a mean depth of coverage of 31.96 and 32.88 for the AAA and normal samples, respectively. We identified 203 somatic variants and confirmed 34 protein-altering somatic mutations in 25 genes. Among the confirmed variants, 11 mutations were not reported in the dbSNP database before. According to the literature review, none of these 25 genes were reportedly associated with AAA.

CONCLUSIONS: Our findings here may provide potential targets for effective prevention of human AAA development and progression.

Key words:
Abdominal aortic aneurysm, Exome sequencing, Gene expression profile.

Introduction

Abdominal aortic aneurysm (AAA), is a complex multifactorial disease with life-threatening implications, accounting for nearly 13,000 deaths in the United States in 2007[1]. Characteristics of AAA pathogenesis include inflammation, extracellular matrix degradation, vascular smooth muscle cell apoptosis and oxidative stress[2]. Multiple genetic and environmental risk factors are involved in AAA formation and progression. Although population based screening with abdominal ultrasound scan reduces AAA related death rate, the evidence base of pharmacological therapies to attenuate AAA progression is wanting. Understanding the genetic architecture of AAA helps capture its characteristics and may provide potential targets for the inhibition of AAA progression, offering more options rather than surgery for the patients.

Previous association studies or genome-wide gene expression profile analysis have proposed many genes[3] or variants[4] involved in AAA pathogenesis, such as genes related with extracellular matrix (ECM)[5,6], cardiovascular system[7], immune system[8], signaling pathways[9], cell growth and cell survival[10]. However, common association studies typically focus on associations between genetic variants and traits like major diseases, no further investigation of gene expression on the transcription level is involved. Meanwhile, expression profile analysis usually focuses on differentially expressed genes (DEGs) only without considering the underlying causative variants. Thus, genetic factors contribute to the etiology of AAA is now still poorly understood.

Current available high throughput experimental strategies such as microarray and sequencing have offered great ease for researchers to explore underlying mechanisms for complex disease. Here to search for the pathogenesis of AAA, firstly, we performed whole exome next generation sequencing using DNA obtained from AAA tissue and normal abdominal aorta of one male patient to explore the genes with key somatic variants; secondly, gene expression analysis with data downloaded from the Gene Expression Omnibus (GEO) database was also carried out to further explore the underlying pathogenesis. Our findings here would provide novel insights into the pathogenesis of AAA, constituting a great advance for the development or improvement of therapies to attenuate AAA progression.

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Materials and Methods

Sample description

This study was approved by the Institutional Review Board of Guangdong General Hospital. Signed informed consent was obtained from the patients before he entered the study. Information about medical history and family history was also acquired.

Full thickness aortic wall tissue specimens of AAA and adjacent normal abdominal aorta were obtained from a 72 years old male patient. The patient was diagnosed with AAA and underwent curative surgical procedure at Guangdong General Hospital. The aneurysm located below the renal arteries. Using 64 multi-detectors helical computed tomography angiography, the maximum diameter of AAA was measured as 63 mm. AAA tissues from the maximum size of dilation were used for subsequent exome sequencing. No alcohol drinking history was reported. The patient has been smoking for almost 40 years (15 cigarettes per day). Besides, the patient was also suffered from hypertension, Atenolol (3 times per day, 25 mg each time) and hydrochlorothiazide (2 times per day, 25mg each time) were taken to maintain the blood pressure as 145/95 mmHg. No other co-morbidities such as diabetes mellitus or arteritis were diagnosed.

Target sequence enrichment and sequencing

DNA of both tissues was extracted using traditional phenol chloroform extraction method. Enrichment of coding exome and flanking intronic regions for both DNA samples was performed using a solution hybrid selection method with the SureSelect® human all exon 38M kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's standard protocols. Briefly, DNA was first sheared by sonication (Bioruptor NGS, Diagenode, Liège, Belgium) to around 325 bp fragment sizes, which were collected using the Minelute Gel Extraction Kit (Qiagen, Hilden, Germany) from electrophoresis gel. The fragment ends were repaired and adaptors were ligated to the fragments, which were then purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The quality of the fragmentation and purification was assessed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The resulting fragments was amplified by PCR and captured by hybridization to the biotinylated RNA library baits. The resulting whole-exome DNA library was quantified with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to check the size distribution before paired-end sequencing with read length of 115 bp on the Illumina Genome Analyzer IIx platform (Illumina, San Diego, CA, USA). Raw sequencing reads were then filtered using fastx tool kit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Image analysis and base calling were carried out using Illumina RTA versions 1.5 and 1.6 respectively with default parameters. Genomic DNA library preparation, target sequence enrichment, and massively parallel sequencing were accomplished by iGenomics Co., Ltd (Guangzhou, China).

Reads alignment and variants detection

Paired-end reads were aligned to the reference human genome (GRCh37.p5) by using the software BWA (Burrows-Wheeler Aligner: version 0.6.1)[11]. First, the reference was indexed by the command 'index'; Second, suffix array coordinates of good hits for each read were found using the command 'aln'; Third, suffix array coordinates were converted to chromosomal coordinates and pair reads with the command 'sampe'. After the alignment, the SAMtools software package (version 0.1.16) was used to remove PCR duplications[12]. In order to get accurate somatic variant call, we performed local realignment around indels using GATK (Genome Analysis Toolkit)[13]. Candidate somatic variants were detected using VarScan (version 2.2.8)[14]. Then, identified variants were filtered by the accessory script (fpfilter.pl, version 1.01) with default parameters[14]. To qualify the identified variants, we reviewed all candidate variants using the Integrative Genomics Viewer[15].

PCR validation

All the protein-altering somatic variants, including nonsynonymous SNVs (Single Nucleotide Variants), variants at splicing sites and exonic insertions and deletions (indels), were validated using PCR and Sanger sequencing. Primers were designed and used to amplify target regions by PCR for genomic resequencing on ABI 3730 sequencers (Life Technologies, Carlsbad, CA, USA). The resulting data were screened for the target variants through manual steps. Genes with confirmed variants were further annotated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Gene expression analysis

Microarray data (GSE7084) for gene expression validation were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). We only used the data sets based on Illumina Sentrix Human-6 Expression BeadChip platform. That's, expression profiles for six cases (abdominal aortic aneurysm), seven controls (abdominal aorta) and one pair (case *vs* control) of pooled samples were included. Entire data sets including matrix files and series matrix files for all samples (ftp://ftp.ncbi.nlm.nih.gov/pub/geo/DATA/SeriesMatrix/GSE7084/) were downloaded. Raw intensity data in the series matrix files were aligned to a common scale with quantile normalization[16] and log2 transformed as expression value. The log2-transformed values for AAA samples and normal samples were stored separately and statistical t tests with multiple test correction (Benjamini and Hochberg) were carried out to detect DEGs. The threshold of significantly expressed genes was set as p-value less than 0.05 in this study. All above procedures were carried out using R statistical software (v2.14.1) with BioConductor, limma packages (3.12.1) and libraries[17]. Expression analysis was carried out for the genes with validated protein-altering variants. To further explore potential underlying pathogenesis, expression analyses for genes which encode proteins directly interact with the product of mutated genes based on the Human Protein Reference Database (http://www.hprd.org/) and the Biomolecular Interaction Network Database (http://bind.ca) were also carried out.

Results

Detail information for the exome sequencing results is listed in Table I. Briefly, a total of 35.13 Mb and 38.12 Mb clean reads were generated for the AAA and normal tissue samples, respectively. Over 99% of the reads were mapped to the whole genome and more than 95% of the mapped reads were properly paired. The duplication rates for AAA and the normal sample were 4.39% and 5.74%. Approximately, a total of 2.44 Gb bases were aligned to the enriched target regions, achieving a mean depth of coverage of 31.96 and 32.88 for AAA and the normal tissue sample, respectively.

According to the variants detection using VarScan[14], a total of 203 somatic variants were detected (Table II and Figure 1). According to the annotation results, 38 of the somatic variants were supposed to be protein-altering, including 35 missense SNVs, 2 SNVs at splicing sites and 1 frameshift deletion. These protein-altering variants were validated using PCR and Sanger sequencing. Taken together, 34 out of 38 (89.47%) variants were confirmed (Table III and Figure 1). All these variants are heterozygote in the AAA sample. Two SNVs locate at the splicing sites of gene *CCDC175* and *RAET1E*. We also found one mutation leading to frameshift in *CEP290*. Other mutations were all annotated as missense SNVs. Eleven mutations were not reported in the dbSNP (Single Nucleotide Polymorphism database) (Version 137, ftp://ftp.ncbi.nih.gov/snp/organisms/human_9606/) before. According to the function prediction results using SIFT (Scale-Invariant Feature Transform)[18] and PROVEAN (Protein

Table I. Summary statistics of AAA and normal abdominal aorta exome sequencing

Samples	AAA	Normal
Clean Reads Number (Mb)	35.13	38.43
Clean data (Gb)	2.93	3.04
Reads mapped to the genome (Mb)	34.86	38.12
Total mapped reads in pairs	33.67	36.71
Data mapped to genome (Gb)	2.91	3.02
Data mapped to target region (Gb)	1.20	1.24
Duplication rate (%)	4.39	5.74
Capture rate (%)	41.30	40.97
Mean depth of target region (×)	31.96	32.88
Coverage of target region (%)	98.81	99.24

Table II. Statistics of the detected somatic variants.

Type	Number
Splicing site	2
UTR5	3
ncRNA-exonic	6
Intergenic	33
Intronic	95
Exonic	51
UTR3	4
Upstream	4
ncRNA-intronic	5
Missense SNV	35
Nonsense SNV	0
Synonymous SNV	15
Frameshift deletion	1

Note: Variants are annotated based on transcripts, that is, one variant may be assigned as different types according to different transcripts. Therefore, overlap exists in different cells of the second column.

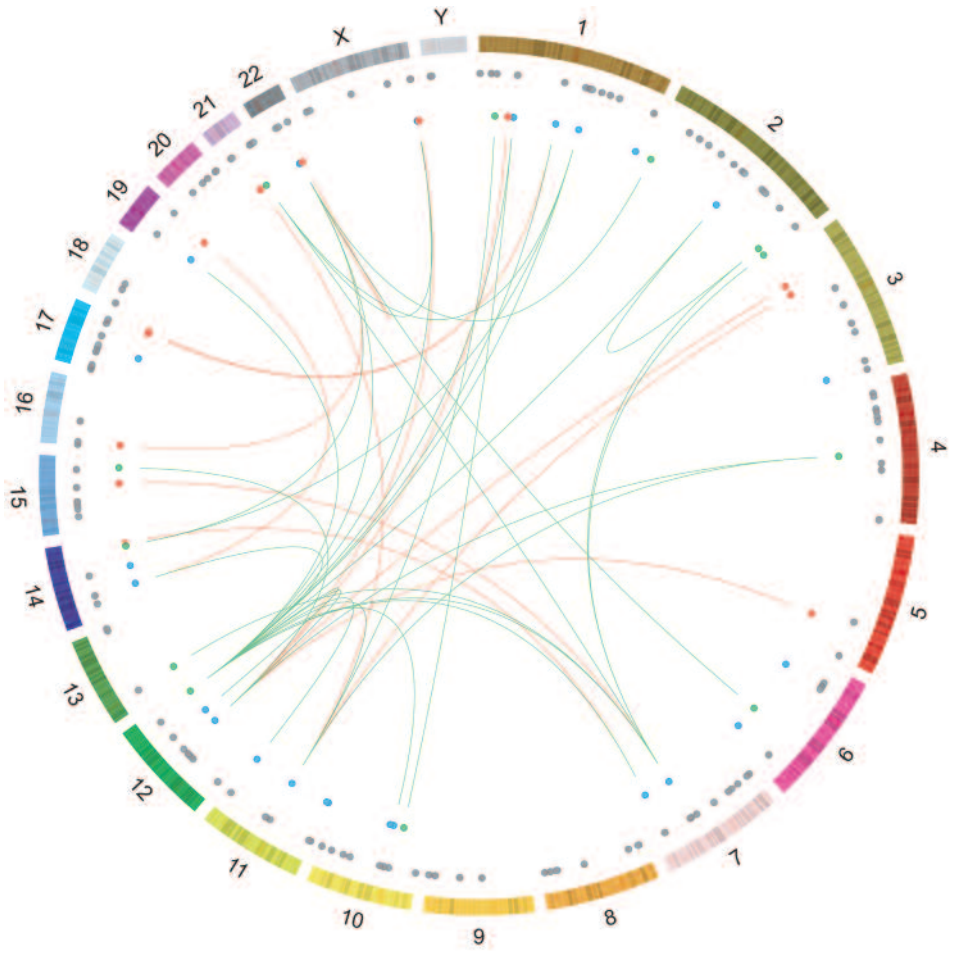

Figure 1. Distribution of the somatic variants across the whole genome. All 203 somatic variants are showed gray dots. Genes with confirmed protein-altering variants are showed in blue dots. Overexpressed genes are showed in red and down-regulated genes are showed in green. The inner lines indicated their direct interaction. Red lines indicate that the mutated genes directly interact with overexpressed genes while green lines indicate that mutated genes directly interact with down-regulated genes.

Variation Effect Analyzer)[19], 13 missense SNVs were predicted to be damaging or deleterious supported by at least one prediction method.

According to the KEGG pathway annotation for the mutated genes, 9 genes were mapped to various pathways (Table IV). Three genes, including *IRAK3*, *PRSS1* and *TUBA3D* were mapped in more than one pathway.

Expression analysis results are showed in Table IV. For the 25 genes with confirmed mutation, 12 of them were not detected in the current expression array. None of the rest 13 genes showed differential expression. However, 27 genes which encode proteins directly interact with the proteins encoded by 16 mutated genes were found to be differentially expressed (Table IV and Figure 1).

Discussion

AAA is a common disorder with an incompletely understood etiology. Here to search for underlying etiology, we performed whole exome sequencing for AAA and adjacent normal abdominal aorta tissue from one male AAA patient. Further gene expression analysis using downloaded dataset from the GEO database was also carried out to explore the underlying molecular mechanisms.

We identified 203 somatic variants and confirmed 34 protein-altering somatic mutations in 25 genes (Table III and Figure 1). According to the literature review, none of these genes were reportedly associated with AAA. However, several genes may be related with the progression of AAA through various cellular processes.

Table III. Information for the validated somatic mutations.

Gene	Chr	Position	Ref	Alter	Ref-AA	Alt-AA	Type	SIFT	PROVEAN	dbSNP137
AGAP7	10	51465913	G	T	N	K	Missense	Neutral	Tolerated	rs4043621
ANKLE1	19	17394637	A	C	H	P	Missense	Neutral	Tolerated	rs201201842
BARX2	11	129321248	C	T	P	L	Missense	Neutral	Tolerated	rs146199848
CABIN1	22	24494089	A	C	T	P	Missense	Neutral	Damaging	rs77920906
CCDC175	14	60007391	A	T	-	-	Splicing	-	-	NA
CEP290	12	88512305	T	.	I	-	Frameshift	-	-	NA
CHRNA10	11	3690534	T	C	E	G	Missense	Neutral	Tolerated	rs77958837
ESRRA	11	64083331	C	T	R	C	Missense	Deleterious	Damaging	rs80310817
HEATR5A	14	31819047	C	T	V	M	Missense	Neutral	Damaging	rs3736918
IRAK3	12	66605228	A	G	I	V	Missense	Neutral	Tolerated	rs1152888
KCNN3	1	154842199	G	T	P	Q	Missense	Neutral	Tolerated	NA
LHX1	17	35298121	G	C	K	N	Missense	Deleterious	Damaging	rs200637129
MAGEA10	X	151303236	A	C	V	G	Missense	Deleterious	Damaging	rs201253472
MARCH8	10	45956819	T	G	T	P	Missense	Deleterious	Damaging	NA
MUC21	6	30955160	G	A	S	N	Missense	Neutral	Tolerated	rs77947051
MUC4	3	195505849	G	A	A	V	Missense	Neutral	Tolerated	NA
MUC4	3	195506617	G	A	A	V	Missense	Neutral	Tolerated	rs199757719
MUC4	3	195507323	T	C	T	A	Missense	Neutral	Tolerated	rs201618076
MUC4	3	195507332	A	T	S	T	Missense	Neutral	Tolerated	rs200058677
MUC4	3	195510326	C	T	D	N	Missense	Neutral	Damaging	NA
MUC6	11	1017591	C	G	R	P	Missense	Neutral	Tolerated	NA
MUC6	11	1017595	A	C	S	A	Missense	Neutral	Tolerated	rs111641154
MUC6	11	1018279	C	T	G	R	Missense	Neutral	Damaging	rs201852389
MUC6	11	1018329	T	A	K	M	Missense	Neutral	Damaging	rs75968137
MUC6	11	1018341	G	A	P	L	Missense	Neutral	Damaging	rs79748612
MUC6	11	1018483	C	G	E	Q	Missense	Neutral	Tolerated	rs78265558
OR2T8	1	248084909	T	G	M	R	Missense	Deleterious	Damaging	rs34508376
PASD1	X	150791434	G	T	L	F	Missense	Neutral	Tolerated	rs201964546
PRSS1	7	142459785	G	T	A	S	Missense	Neutral	Tolerated	NA
RAET1E	6	150209805	T	A	-	-	Splicing	-	-	NA
SELRC1	1	53158521	T	C	D	G	Missense	Deleterious	Damaging	NA
SRRT	7	100481800	T	G	F	V	Missense	Neutral	Tolerated	rs201173432
TUBA3D	2	132237732	C	T	R	W	Missense	Deleterious	-	rs145612794
WDR3	1	118497971	A	G	I	V	Missense	Neutral	Tolerated	NA

Table IV. Mutated pathways in the progression of abdominal aortic aneurysm

Mutated genes	Pathways (ID: description)
CHRNA10	hsa04080: Neuroactive ligand-receptor interaction
HS6ST1	hsa00534: Glycosaminoglycan biosynthesis - heparan sulfate
IRAK3	hsa04210: Apoptosis; hsa04722: Neurotrophin signaling pathway
KCNN3	hsa04911: Insulin secretion
OR2T8	hsa04740: Olfactory transduction
PRSS1	hsa04080: Neuroactive ligand-receptor interaction; hsa04972: Pancreatic secretion; hsa04974: Protein digestion and absorption; hsa05164: Influenza A
RAET1E	hsa04650: Natural killer cell mediated cytotoxicity
TUBA3D	hsa04145: Phagosome; hsa04540: Gap junction; hsa05130: Pathogenic Escherichia coli infection
WDR3	hsa03008: Ribosome biogenesis in eukaryotes

First, a missense mutation in *ESRRA* (estrogen-related receptor alpha) changed the amino acid from arginine to cysteine. Both function prediction methods showed that this mutation was deleterious. ESRRA is a vascular endothelial growth factor (VEGF) regulator[20]. VEGF is known to be a regulator of angiogenesis and may play important roles in aneurysm formation[21, 22] since AAA development is associated with increased angiogenesis. Thus, mutation of ESRRA may interrupt the regular process of angiogenesis, leading to the development of AAA. Besides, although differential expression of ESRRA was not detected, several genes which encode proteins directly interact with the ESRRA protein were differentially expressed. For example, PRKCD (Protein kinase C delta type) was up regulated with the fold change of 1.02 (p = 0.0163, Table V). Previous study reported that PRKCD regulates the stimulation of VEGF mRNA translation[22]. Up regulation of PRKCD may trigger the over expression of VEGF (fold change of 0.69, p = 0.021), contributing to the progression of AAA.

Second, a novel missense mutation in *KCNN3* (Potassium intermediate small conductance calcium-activated channel, subfamily N, member 3) caused the amino acid change from proline to glutamine. This mutation has not been reported in the dbSNP database before. Previous report showed that genetic deficit of *KCNN3* disrupted the endothelium-derived hyperpolarizing factor vasodilator pathway and caused hypertension[23]. Our patient here also suffered from hypertension, taking Atenolol and hydrochlorothiazide daily to keep blood pressure in control. Mutated *KCNN3* may contribute to the development of hypertension here. Hypertension is known to be risk factor of AAA; it is possible that genetic mutations caused hypertensions, which further contribute to the development of AAA. Further investigation of this gene is warranted to declare its contribution to AAA.

In addition, a novel mutation at the splicing site of *RAET1E* (retinoic acid early transcript 1E) was detected. This gene is involved in the natural killer cell mediated cytotoxicity pathway (Table IV), which belongs to the immune system. Detection of mutated immune pathway in AAA is not unexpected since autoimmunity has been proposed as one of the pathogenesis[24] and immune involvement has been implicated in AAA [25-27].

Protein-altering mutations were also confirmed in two mucin genes, *MUC4* and *MUC6*. Previous studies have proposed that nicotine/cigarette smoke promoted metastasis of pancreatic cancer through up regulating MUC4 expression[28]. Our patient has been smoking for almost 40 years and smoking is one of the known risk factors of AAA. Whether cigarette smoking induces AAA through its correlation with *MUC4* needs further investigation.

Conclusions

We performed exome sequencing for AAA and adjacent normal abdominal aorta tissue from one male AAA patient to identify keys somatic variants which may contribute to the development of AAA. We identified 38 protein-altering somatic mutations and confirmed 34 of them in 25 genes. Our findings here may offer potential targets for the development of therapeutic options for the attenuation of AAA progression.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

Table V. Results of gene expression analysis

Mutated Gene	*p*-value[a]	Genes encode proteins directly interact with the products of mutated gene	*p*-value[b]
AGAP7	-	-	-
ANKLE1	-	*UBC*	3.10E-02
BARX2	0.25	*UBC*	3.10E-02
CABIN1	0.40	*HDAC2*	4.23E-04
		AP1B1	8.02E-04
		YWHAQ	5.17E-03
		CALM1	1.98E-02
		UBC	3.10E-02
		UBN1	4.39E-02
CCDC175	-	-	-
CEP290	-	*UBC*	3.10E-02
CHR-10	0.86	-	-
ESRRA	0.18	*ADH5*	9.20E-06
		PNRC2	1.50E-02
		PRKCD	1.63E-02
		NRIP1	2.10E-02
		UBC	3.10E-02
HEATR5A	-	*NUP62*	1.20E-02
		UBC	3.10E-02
IRAK3	0.86	*ADH1B*	1.12E-04
		CD14	4.59E-04
		NTRK3	4.61E-04
		IRAK1	8.37E-04
		ATP6V0B	1.43E-03
		MYD88	2.50E-03
		UBC	3.10E-02
KCNN3	0.11	*APP*	8.14E-03
		CALM1	1.98E-02
		UBC	3.10E-02
LHX1	0.72	-	-
MAGEA10	0.74	*UBC*	3.10E-02
MARCH8	-	*UBC*	3.10E-02
MUC21	-	-	-
MUC4	0.92	-	-
MUC6	0.73	-	-
OR2T8	-	-	-
PASD1	-	*APP*	8.14E-03
PRSS1	0.64	*SERPI-1*	4.66E-04
		UBC	3.10E-02
RAET1E	0.72	-	-
SELRC1	-	*SVIL*	3.33E-04
		UBC	3.10E-02
		SELENBP1	5.04E-02
SRRT	-	*APP*	8.14E-03
		SAP18	1.93E-02
		CUL3	2.14E-02
		KIAA0101	2.36E-02
		COPS8	2.54E-02
		UBC	3.10E-02
TUBA3D	-	*CUL3*	2.14E-02
		UBC	3.10E-02
WDR3	0.66	*SIRT7*	6.81E-03
		USP36	1.10E-02
		UBC	3.10E-02

For the genes whose products directly interact with the proteins encoded by the mutated genes, only the differentially expressed ones are shown. [a]*p*-value for the mutated genes; [b]*p*-value for the genes which encode proteins directly interact with the proteins of the mutated genes.

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